Genotyping analysis and functional characterization of CYP2D6 variants found in the Guatemalan, Kenyan and Vanuatu populations（グアテマラ、バヌアツ及びケニア人集団における薬物代謝酵素CYP2D6の遺伝的多様性に関する研究）

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グアテマラ、バヌアツ及びケニア人集団における薬物代謝酵素 CYP2D6 の遺伝的多様性に関する研究

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The Cytochrome P450 2D6 (*CYP2D6*) gene, currently one of the most extensively characterized P450 isoenzymes, has an intrinsic role in the metabolism of 25% of all clinically relevant drugs, including tricyclic antidepressants, beta blockers, and opiates. CYP2D6 enzyme function is known to vary depending on individual genotypes. Significant racial differences in site and frequency of genetic polymorphisms, cause differential enzyme activity profiles among worldwide populations.

The phenotypic consequences resulting in the presence of genetic polymorphisms critically affects the biotransformation of CYP2D6 substrates either in an increase, decrease, or complete loss of enzymatic activity. In this sense, the phenotype–genotype refers to the relationship between the measurement of the actual hydroxylating capacity (metabolic phenotype) and the estimation based on the activity score of the alleles. In the case of poor metabolizers, the expected correlation with the metabolic phenotype is practically achieved as the allelic variants result in an enzyme with null activity; either by deletion of part of the gene, splicing defects or displacement of the reading frame. Conversely, normal or intermediate metabolizers do not always correspond, omitting considerations for misclassification, additional environmental factors such as concomitant pharmacological treatment, disease or dietary interactions can intervene in the actual metabolic activity producing a phenocopy.

While *CYP2D6* allele and phenotype frequencies have been extensively studied, currently, very little ethnically specific data is available regarding admixed populations in the Central American, East African and South Pacific region, including Guatemala, Kenya, and Vanuatu. Moreover, to our knowledge, P450 genotyping information is not yet available for the Guatemalan population in general or self-defined ethnically distinct groups. Our study populations are the product of complex admixture; thus, it is of interest to evaluate the interethnic variability of polymorphisms with pharmacogenetic relevance, including *CYP2D6*.

Given the scarceness of CYP2D6 related data in these populations, the purpose of this study was to perform a pharmacogenomic analysis of the Guatemalan, Kenyan and Ni-Vanuatu populations using PCR gene amplification coupled with Sanger sequencing-based SNP genotyping. Additionally, ddPCR based methodologies were used to verify detected copy number variations, including gene deletions, multiplication, and tandem arrangement genes. The activity score system model A was used to determine *CYP2D6* phenotype assignments from genotype data. And ultimately characterize the enzymatic properties of novel CYP2D6 variant proteins expressed in 293FT cells in vitro by measuring Dextromethorphan O-demethylation activity, a drug classified as a synthetic analog of codeine and a commonly used probe substrate for determining CYP2D6 activity, in which CYP2D6 primarily mediates the formation of its metabolite dextrorphan from dextromethorphan via O-demethylation with a minor contribution from CYP3A4. Moreover, in an attempt to elucidate the molecular causes and structural alterations rationalizing the observed functional effects fitting to each variant, a 3D structural docking analysis was performed in which the molecular interactions between CYP2D6 novel variants and dextromethorphan were evaluated.

This study revealed significant differences in the frequency *CYP2D6* alleles and the resulting metabolic phenotypes among the study populations. Being a marked prevalence of functional alleles in all studied populations, with the majority of population subjects classified as normal metabolizers, with frequencies exceeding 60% in all populations (Guatemala 69.4%, Vanuatu 89.2%, and Kenya 64.1%,). Correspondingly, a low frequency for decreased function defining genotypes was observed in the Guatemalan and Ni-Vanuatu population, while approximately 36% of Kenyan subjects presented substrate-dependent reduced function alleles. These data were consistent with previous studies, in which approximately 50% of CYPD6 alleles found were normal function in Americans, Hispanics, Africans, and African Americans. while Oceanians are known to exhibit only full-functional variants with frequencies of wild-type *CYP2D6* of up to 85%, concurring with the findings for the Ni-Vanuatu population

Accordingly, the main allelic frequencies in Guatemala and extrapolated phenotypes are in accordance with Amerindian populations found in North and Central America but differ considerably from those in South America and the Caribbean. These differences are considered in part to be caused by the higher degree of African admixture in which genotypic characteristics are more profoundly influenced by polymorphisms native to this region in which metabolizer status has been observed to be influenced in great part by the high frequency of the decreased function alleles. Whereas the allelic frequencies and extrapolated phenotypes for Kenya and Vanuatu do not notably differ from populations found in their respective regions. Notably, the A449D polymorphism was detected in the Guatemalan population, with a frequency of approximately 5%; this polymorphism has been reported only once before in a

study comprising three populations of Mexican Amerindians with an approximate frequency of 4%, this finding further supports anthropological theories of ancient migratory patterns between neighboring Amerindian populations.

Additionally, a total of 17 novel polymorphism were found (Guatemala: A449D, Y335C, M279K, V307I, P354S, A165V, D337N. R329C, S304L, R344X; Vanuatu: V104A, P171L, R269Q, G306R, V402L, and Kenya: L203F, I339L, H376R) The analysis of novel non-synonymous SNPs identified amongst Guatemalan subjects in respects to the impact of their amino acid substitutions on protein structure/function revealed that A449D and P354S, are estimated to detrimentally affect enzymatic function and resultant metabolic activity. While amongst the Ni-Vanuatu and Kenyan functionally characterized variants, 3 novel variants (P171L, G306R, V402L) and 3 variant containing alleles (L203F, I339L, H376R) respectively, showed significantly reduced intrinsic clearance compared to that of wild-type CYP2D6.1.

The A449D a variant found as an additional SNP located in exon 9 of the *CYP2D6*4* allele, is a substitution of hydrophobic alanine with neutral aspartic acid in the L helix. This change would decrease the hydrophobicity and integrity of this helix causing it to shift outwards and gain additional interactions with substrate recognition sites (SRS) and the active cavity indirectly influencing heme incorporation.

Located in the K helix, the P354S substitution would be expected to affect this highly conserved turn motif by the presence of additional heme interactions with SRS-1 member F120, involved in substrate recognition and binding while also lacking interactions with the E334 residue. Taken together, these changes could perturb the active site and influence heme incorporation, resulting in a protein with low substrate affinity and enzyme efficiency.

3D structural analysis further revealed that variants with reduced enzymatic activity showed that the amino acid substitutions present caused shifts in molecular interactions resulting in structural changes involving access channels, the heme binding site, and substrate recognition sites. Correspondingly, the determination of microsomal P450 contents by CO difference spectroscopy revealed that possible structural differences affecting heme incorporation in the G306R variant and L203F containing allele as they did not show an increase in absorbance near 450nm, signifying the amount of active holoprotein was insufficient for detection.

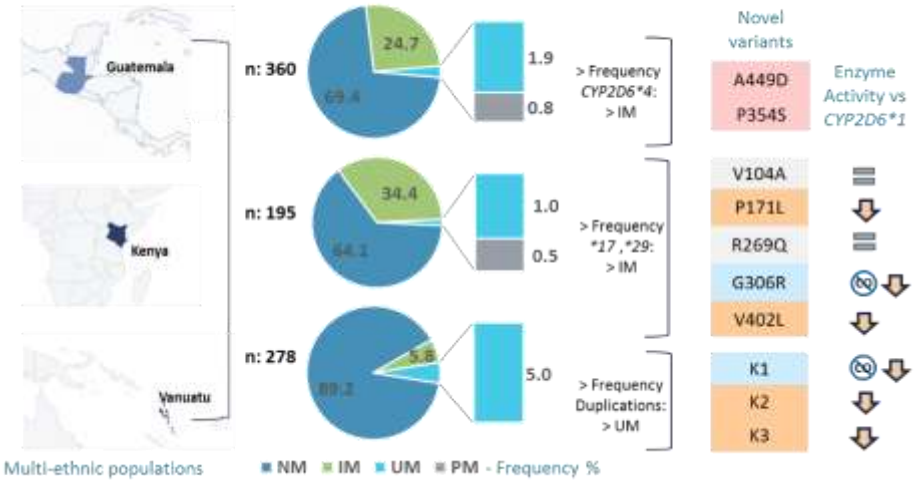
Amino acid substitutions in the conserved G306, located in the N-terminus of the I helix and present in the SRS-4, can lead to increases in the side-chain volume introducing steric clashes and thus obstruct the binding mode. Several structural changes were observed in the K1 variant, harboring the L203F, and *CYP2D6*2* (R296C and S486T) substitutions, including a decrease in distance between the F203 and amino acids located in the vicinity of the solvent channel hydrophilic bottleneck. These changes, in turn, lead to the decreased interactions

with substrate recognition sites and changes in heme binding patterns that might result in a structurally unstable holoprotein and thus affect overall function.

Moreover, the V402L variant and H376R containing novel allele K3, which exhibited significantly decreased activity but detectable P450 content, both showed interactions that may result in impediments with substrate recognition. The V402L substitution found in the K' helix was seen to lack alkyl interactions between the substrate and substrate recognition site members. While at the same time causing several modified interactions involving heme. Taken together, these changes could generate continuous structural differences that would influence heme stereoselectivity, resulting in decreased substrate recognition. This theory is further supported by the considerable increase in K_m observed for this variant, when compared to that of wild-type.

Lastly, the K3 variant found to have the *CYP2D6*10* substitution P34S alongside S486T and the novel H376R located in the β-strand and SRS-5. In this variant, substitution with the positively-charged polar arginine causes the β-strand to shift towards the external part of the protein, which in turn, causes several observed functionally important structural changes. These conformational changes could cause weak interaction with the active site and binding pocket. As a result, potentially leading to poor substrate recognition, which is consistent with these variants increased K_m and V_{max}.

Allelic frequency determination, polymorphism identification in the CYPD6 gene and its subsequent evaluation over enzymatic activity performed in this study could aid in building population specific therapeutic profiles which in turn would benefit from taking ethnic differentiation and intra-ethnic variations into consideration. And as a result, improve therapeutic outcomes as well as supporting clinical pharmacologic profiling efforts.



別紙様式（Ａ４判）　　論 文 審 査 結 果 の 要 旨

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論 文 題 目：Genotyping analysis and functional characterization of CYP2D6 variants found in the Guatemalan, Kenyan and Vanuatu populations（グアテマラ、バヌアツ及びケニア人集団における薬物代謝酵素 CYP2D6 の遺伝的多様性に関する研究）

マラリアは、ハマダラカにより媒介される原虫感染症であり、赤道周辺地域を中心に年間 2 億人以上が罹患し、約 42 万人が死亡している。ヒトに感染するマラリア原虫として、熱帯熱マラリア、三日熱マラリア、卵形マラリア、四日熱マラリア及びサルマラリアの 5 種が報告されているが、三日熱マラリア及び卵形マラリアに感染した場合、これらの原虫の一部はヒトの肝臓内で肝休眠体を形成する。肝休眠体は活動を休止した原虫の形態であり、数週間から数か月の後に再び活性化し、ヒト赤血球内で原虫が増殖することによりマラリア再発を引き起こす。抗マラリア薬プリマキンは、現在使用されている薬剤の中で唯一肝休眠体を殺滅可能であり、マラリア根治療法に用いられている。

プリマキンは、ヒトの肝臓内において薬物代謝酵素チトクローム P450（CYP）により代謝活性化を受け、肝休眠体殺滅効果を示すプロドラッグである。その代謝物の中でも、5-ヒドロキシ体は非常に不安定であり、体内で直ちに酸化され 5,6-オルトキノン体となる。このときに生成される活性酸素種が細胞に酸化ストレスを与えることが示唆されていることから、5-ヒドロキシ体の生成はプリマキンの薬効発現に重要と考えられる。近年、プリマキン代謝酵素である CYP2D6 の遺伝的低酵素活性者では薬効が得られず、マラリア再発率が高いことが報告されている。したがって、CYP2D6 の遺伝型を考慮した抗マラリア薬の選択や投与量調整は効果的なマラリア治療を展開する上で極めて重要である。しかし、中央アメリカ、南太平洋、アフリカ地域などのマラリア流行地域住民における CYP2D6 遺伝子多型の部位、頻度、酵素活性への影響についてはほとんど明らかになっていない。

本論文では、プリマキンの代謝活性化に関与する CYP2D6 に着目し、マラリア流行地域であるグアテマラ、バヌアツ及びケニア人集団における CYP2D6 の遺伝的多様性を明らかにすることを目的にしている。さらに、各集団において同定された CYP2D6 遺伝子多型に対して、それらに由来するアミノ酸置換導入 CYP2D6 バリアント酵素の機能変化を明らかにするため、ヒト胎児腎臓由来 293FT 細胞に発現させた組換え酵素を CYP2D6 の特異的基質薬物であるデキストロメトルファンと反応させ、その代謝物生成量から酵素機能変化を評価している。

その結果、アミノ酸置換を誘発する新規遺伝子多型を 17 種類同定し、それらの機能変化を詳細に明らかにした。特に、酵素活性が消失するバリアントに関しては、プリマキンを基質とした場合、活性代謝物が体内で産生できずに薬効が得られない可能性が高い。したがって、本論文は、遺伝的背景から個々に最適な抗マラリア薬の投与量を選択をする上で極めて重要な知見を含んでいる。

よって、本論文は博士（薬科学）の学位論文として合格と認める。